



## Review

# Arginine-rich cell-penetrating peptides

Nathan Schmidt<sup>a,1</sup>, Abhijit Mishra<sup>b,1</sup>, Ghee Hwee Lai<sup>a,1</sup>, Gerard C.L. Wong<sup>a,b,\*</sup>

<sup>a</sup> Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States

<sup>b</sup> Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States

## ARTICLE INFO

### Article history:

Received 1 October 2009

Revised 9 November 2009

Accepted 11 November 2009

Available online 16 November 2009

Edited by Wilhelm Just

### Keywords:

TAT

Polyarginine

Cell-penetrating peptides

Protein transduction domain

Membranes

Drug delivery

## ABSTRACT

**Arginine-rich cell-penetrating peptides are short cationic peptides capable of traversing the plasma membranes of eukaryotic cells. While successful intracellular delivery of many biologically active macromolecules has been accomplished using these peptides, their mechanisms of cell entry are still under investigation. Recent dialogue has centered on a debate over the roles that direct translocation and endocytotic pathways play in internalization of cell-penetrating peptides. In this paper, we review the evidence for the broad range of proposed mechanisms, and show that each distinct process requires negative Gaussian membrane curvature as a necessary condition. Generation of negative Gaussian curvature by cell-penetrating peptides is directly related to their arginine content. We illustrate these concepts using HIV TAT as an example.**

© 2009 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

## 1. Introduction

Cellular uptake of biologically active molecules is a major obstacle in pharmaceutical drug design and controlled drug delivery. While a broad range of therapeutic agents, including proteins, peptides and oligonucleotides, have been successfully introduced to target cells using viral vectors [1], and methods such as electroporation, microinjection, and liposome encapsulation [2], these internalization strategies have a number of drawbacks. Problems include inefficient drug delivery, high variability of drug expression among target cells, cellular damage and toxicity, and restrictions based upon drug and cell type. The first barrier to efficient and controlled intracellular delivery is the plasma membrane which prevents direct translocation of hydrophilic macromolecules. In vivo, the most common pathway for bringing a macromolecule into a cell is through endocytosis. However, the fate of an endocytosed macromolecule is unpredictable; it may remain trapped in endosomes and suffer degradation by the acidic pH and digestive enzymes.

\* Corresponding author. Present address: Department of Bioengineering, University of California, Los Angeles, 5121 Engineering V, Los Angeles, CA 90095, United States. Fax: +1 310 794 5956.

E-mail address: [gclwong@illinois.edu](mailto:gclwong@illinois.edu) (G.C.L. Wong).

<sup>1</sup> Present address: Department of Bioengineering, UCLA, Los Angeles, CA 90095, United States.

Biology has evolved ways to circumvent these problems as a number of proteins are permeable to mammalian cell membranes. This ability is conferred by a localized region in the protein known as the protein transduction domain. Furthermore, the isolated peptide sequence, sometimes referred to as a cell-penetrating peptide, retains the transduction properties of the native protein. These cell-penetrating peptides comprise a class of short (<20 amino acid) cationic peptides that have the ability to traverse the cell membranes of many different types of mammalian cells. A wide variety of macromolecules have been attached to these peptides and subsequently internalized. Moreover, after uptake the cargo maintains its activity. The ability of cell-penetrating peptides to translocate biologically active molecules into cells makes these peptides promising candidates for drug delivery applications.

Among the cell-penetrating peptides, the arginine-rich cell-penetrating peptides have been the most widely studied [3,4]. Examples include the TAT peptide from the HIV transactivator protein TAT, Penetratin, a 16 amino acid domain from the Antennapedia protein of *Drosophila*, a flock house virus (FHV) coat peptide (sequence 35–49), and oligoarginines [3,5]. In this review, we focus on TAT peptide, partly because it has attracted the most attention, but also because it is a prototypical example that has many of the essential characteristics of the arginine-rich cell-penetrating peptides. This is not to say all cell-penetrating peptides behave in the exact same manner, nor do they exhibit identical activity profiles. Rather we propose that the structure function relationships

observed in the TAT peptide may apply to similarly structured arginine-rich peptides.

## 2. TAT as a prototypical example of a cell-penetrating peptide

In 1988, Green and Lowenstein [6], and Frankel and Pabo [7], independently discovered that the transactivator of transcription (TAT) protein of the Human Immunodeficiency Virus can penetrate cells and activate the viral genome replication. The TAT protein is an 86 amino acid long protein that is released by infected cells and is an essential regulatory gene for HIV replication [8]. In 1997, Vives et al. [9] found that a 11-amino acid sequence, TAT (47–57), now known as the TAT peptide or TAT PTD, can not only enter cells but is more efficient than the full length protein. It was observed that the chirality of the peptide backbone has no effect on cellular uptake of TAT peptide; inverse and retro forms were able to enter cells as efficiently as the native peptide, suggesting uptake does not require a specific binding site. The TAT peptide can enter cells efficiently, either alone or linked to macromolecules like proteins, oligonucleotides or liposomes. TAT-mediated delivery appears to be independent of cargo size. Proteins in excess of 100 000 Da, 40 nm nanoparticles and even 200 nm liposomes have been delivered inside cells using TAT peptide. The liposomes were intact inside the cells and remained so even 1 h after transduction. Conversely, non-conjugated proteins in the incubation media were not able to enter cells [10].

TAT peptide is highly cationic with 6 arginine and 2 lysine residues. Substitution of any basic residue with neutral alanine reduces activity, while substitution of neutral residues has no effect, implying the net positive charge of TAT is necessary for cellular uptake. It has been hypothesized that the utility of being positively charged likely comes from the resulting strong electrostatic interactions with the plasma membranes of eukaryotic cells. Studies on the binding affinities of cationic cell-penetrating peptides indicate these peptides strongly bind electrostatically to the various anionic species present at the extracellular surface of cell membranes, including lipid head groups, proteins like nucleolin, and proteoglycans such as heparin sulfate [11,12].

Although electrostatic interactions are known to be important for arginine-rich cell-penetrating peptides, non-electrostatic effects such as hydrophobicity and peptide structural transitions can also contribute to the binding affinity of amphipathic cell-penetrating peptides to cell membranes [13]. For example, Penetratin adopts a random coil structure in solution, and transforms to an  $\alpha$ -helical conformation at high lipid to peptide molar ratios [14,15]. Moreover, decreasing the lipid to peptide ratio promotes a higher degree of  $\beta$ -sheet conformation [15]. Both of these secondary structural transitions increase the amphipathicity of Penetratin, allowing its hydrophobic moieties to directly interact with the non-polar interior of the lipid membrane, and several studies have implicated insertion of the hydrophobic portions of Penetratin into the membrane as being important for uptake [16–18]. In contrast, the non-amphipathic TAT peptide is unstructured both in solution and when associated with lipid membranes [19], and TAT associates with the membrane surface since hydrophobic interaction is negligible [11].

It is empirically known that the cationic nature of the peptide is a necessary condition but not a sufficient condition for translocation activity. Although, arginine-rich oligomers can enter cells, similar length polymers of other basic amino acids, lysine, ornithine or histidine, cannot [20]. Branched chain arginine polymer is as efficient as the corresponding linear polymer. However, peptide length is an important factor [21]. The efficiency of cellular uptake depends on the number of arginine residues. Arginine polymers with less than five amino acids are not as effective as

polymers with six or more amino acids. Uptake efficiency increases as the peptide length increases up to 15 amino acids. Peptides with more than 15 arginine residues can still enter cells but with significantly less efficiency [20,22]. The guanidinium head group of arginine is the central structural feature required for peptide uptake. Heptamers of citrulline, an isostere of arginine with a nitrogen of guanidine replaced by oxygen, are unable to enter cells [20].

The discovery that the guanidinium residues of arginine are the essential ingredients of a peptide's ability to enter cells has allowed for the design of a range of guanidinium-rich synthetic analogs. Oligoarginine peptoids that have the side chain attached to nitrogen instead of carbon have proved to be more efficient than oligoarginines [21]. Guanidinium-rich oligocarbamates were taken up into cells about three times faster than TAT peptide [23]. Polyguanidino dendrimers, based on diamino acid monomeric units, have also proven to be effective at entering cells [24]. Carbohydrate-based polymers like the guanidinylated neomycin can not only enter cells but can carry large (>300 kDa) bioactive macromolecules along [25]. Recently, Deming and co-workers [26] combined liposome drug delivery system with cell-penetrating peptides by preparing polyarginine–polyleucine block copolymers that self-assemble into vesicles. The vesicles remained intact inside the cells showing their potential to carry large cargoes. Although long polyarginine chains (>20) are thought to be less efficient for intracellular delivery, they facilitated cellular uptake of the vesicles.

## 3. Direct translocation, endocytosis: an either/or discourse?

The exact molecular mechanism of cellular entry of arginine-rich cell-penetrating peptides is currently not fully understood. Initial studies indicated a direct translocation mechanism across the cell membrane that bypassed endocytosis. Fluorescence microscopy and fluorescence activated cell sorting (FACS) studies on cells incubated with fluorescently-labeled peptides showed rapid translocation that was not inhibited when cells were incubated at 4 °C. Addition of metabolic or endocytosis inhibitors also seemed to have no effect on cellular internalization. These experiments, along with the finding that inverse and retro forms of the peptide are as effective, led to the belief that cellular uptake involved an energy-independent, non-endocytotic process that was receptor independent [4,9,10,20].

Most of these early experiments were conducted using microscopy or flow cytometry on fixed cells. In 2003, Richard et al. [27] showed that a mild fixation of cells with formaldehyde drastically changed the intracellular distribution of TAT peptide. Fixed cells showed nuclear localization of TAT peptide while unfixed cells had the peptide located in cytoplasmic vesicles. Additionally, it was shown that flow cytometry was unable to distinguish between membrane-bound and internalized fluorochrome. In living, non-fixed cells analyzed with FACS, a large fraction of the fluorescent peptide was associated with the outer leaflet of the cell membrane instead of being present within the cytoplasm. They demonstrated that trypsin treatment of cells removed surface-bound peptide by digesting the peptide. FACS analysis following trypsin treatment indicated a relatively slow rate of uptake, comparable to that of classical markers of endocytosis. Since then, many other studies have also observed inhibition of cellular uptake at 4 °C and with chemical means that induce energy depletion, indicating an energy-dependent process as the major route for the internalization of cell-penetrating peptides [27–33].

Many groups have proposed that cell membrane heparan sulfate proteoglycans (HSPGs) act as receptors for extracellular TAT uptake. Proteoglycans are negatively charged, and are present on the surface of many cell types. Thermodynamic studies have shown that TAT binds with significantly greater affinity to heparin

sulfate than to anionic lipid vesicles [11,12]. That TAT can interact with different components of the membrane suggests that multiple mechanisms are possible. Moreover, it has been observed that interaction with the cell-penetrating peptides results in aggregation of both anionic lipids and proteoglycans, which may provide further clues for the nature of the alternate mechanisms [34,35].

Ligands that bind to proteoglycans can be internalized through an endocytotic pathway. Both TAT protein and TAT peptide were shown to bind strongly to heparin, a sulfated glycosaminoglycan that mimics the heparan sulfate proteoglycans [36,37]. It was also demonstrated that addition of heparin and dextran, another sulfated glycosaminoglycan, inhibits the cellular uptake of TAT peptide [31,38,39]. Treatment of cells with chemicals that eliminate or cleave the HS proteoglycans resulted in a significant decrease in TAT peptide internalization [40]. Studies with mutant cells that are unable to synthesize glycosaminoglycans showed reduced TAT-mediated transport [39,41,42]. These studies suggest that heparan sulfate can act as a receptor for TAT peptide, and constitutes an important pathway for internalization; however, none of the studies have demonstrated complete inhibition of cellular uptake [41]. In addition, polyarginine was able to enter mutant cells that have less than 2% of the wild-type level of heparin sulfate [25]. This suggests the presence of a heparan sulfate-independent pathway in addition to a heparan sulfate-dependent one.

Analysis of peptide uptake by live-cell microscopy has demonstrated the involvement of endocytosis in the cellular internalization of the TAT peptide. Within the broad classification of endocytosis, there are several possible mechanisms of uptake. Studies on TAT peptide uptake in cells with specific endocytotic pathways chemically inhibited have yielded mixed results. Clathrin-mediated endocytosis has been proposed as the primary mechanism of uptake of arginine-rich transporters. Clathrin-mediated endocytosis is the major and best-characterized endocytotic pathway. It involves strong binding of a ligand to a specific cell surface receptor resulting in the clustering of the ligand–receptor complexes in coated pits on the plasma membrane, formed by the assembly of clathrin. The coated pits then invaginate and pinch off from the plasma membrane to form intracellular clathrin-coated vesicles [43]. It has been reported that in HeLa cells, labeled cell-penetrating peptides colocalize with transferrin, a glycoprotein marker for endocytosis [27,44]. Another study demonstrated that TAT uptake in HeLa cells in the presence of chlorpromazine, a known inhibitor of clathrin-mediated endocytotic pathway, resulted in a 50% inhibition of peptide uptake, while incubation in a potassium-free buffer resulted in a 40% decrease, indicating the involvement of clathrin-dependent pathway [33]. However, other studies with fluorescently labeled polyarginine conjugates [30,45] and fusion proteins [41,46] showed that it does not colocalize with transferrin. Another study with a TAT-avidin conjugate showed only a modest decrease in uptake upon treatment with hyperosmolar medium, a condition shown to decrease clathrin-dependent endocytosis [47].

Caveolin-dependent endocytosis, a lipid raft-mediated form of endocytosis has likewise been implicated. Caveolae are small, hydrophobic membrane microdomains that are rich in cholesterol and glycosphingolipids. Ligands associate with the cell membrane and then become trapped in relatively stationary caveolae, which then bud off the membrane and form caveosomes. Cholesterol is required for caveolar uptake and drugs that specifically bind to cholesterol perturb internalization through the caveolae [43]. TAT-GFP in HeLa cells [46] and in CHO-K1 and HL3T1 cells [41] has been shown to colocalize with caveolin-1. Both TAT-rhodamine [45] and TAT-GFP [41] complexes have also been shown to colocalize with cholera toxin, which is known to proceed through a caveolin-dependent pathway. However, nystatin, a compound known to inhibit caveolae formation, and filipin III had little effect on

the uptake of fluorescently labeled TAT into HeLa cells or CHO cells [33].

A number of research groups have proposed macropinocytosis as the mechanism of uptake for cell-penetrating peptides. Macropinocytosis involves the formation of large endocytotic vesicles of irregular size and shape, generated by actin-driven invagination of the plasma membrane. Macropinosomes have no coat and do not concentrate receptors. They vary in size, sometimes being as large as 5  $\mu\text{m}$  in diameter [43]. Studies have shown dose-dependent inhibition of TAT peptide uptake when cells are pretreated with amiloride, an inhibitor of the  $\text{Na}^+/\text{H}^+$  exchange required in macropinocytosis, or cholesterol is removed with  $\beta$ -cyclodextrin [32,48]. Additionally, cytochalasin D, an inhibitor of actin polymerization, and the macropinocytosis inhibitor ethylisopropylamiloride have been shown to significantly suppress uptake of the arginine-rich peptides into HeLa cells [30]. However, Zaro et al. [49] reported that delivery of oligoarginine in HeLa cells was not inhibited by incubation at 16  $^\circ\text{C}$ , or by treatment with amiloride indicating a mechanism different from macropinocytosis.

Although the direct translocation mechanism from earlier studies has been shown to be an artifact of cell fixation and membrane-associated peptide, studies on live, non-fixed cells have indicated the presence of a non-endocytotic mechanism of cellular uptake of cell-penetrating peptides. Maiolo et al. [50] used confocal microscopy to study the cellular distribution of arginine-rich cell-penetrating peptides in live cells. They found that the uptake characteristics were a mixture of punctate and diffuse staining. Incubating the cells at 4  $^\circ\text{C}$  eliminated most of the punctate staining, indicating that it is due to endocytotic uptake. The diffuse staining appeared to be fast and occurred at both 37 and 4  $^\circ\text{C}$ , indicating a second, non-endocytotic mechanism. The diffuse staining was not due to the release of peptide from endosomes as it appeared first and the punctate staining later, and it continued to occur at 4  $^\circ\text{C}$  when endocytosis is inhibited. Several other studies have also reported cellular uptake when the cells have been incubated at 4  $^\circ\text{C}$  [26,51–53]. It has also been observed that blocking specific endocytotic pathways does not affect the ability of TAT peptide to enter cells.

Examination of translocation of arginine-rich cell-penetrating peptides across unilamellar vesicles as a model system has revealed a range of behavior. Several early studies have indicated that the addition of both TAT peptide [11,54] and Penetratin [55,56] do not induce dye leakage and are not directly translocated in lipid vesicles composed mainly of zwitterionic PC and anionic PG and PS lipids. Membrane potential measurements also indicate that lipid bilayers with similar compositions remain intact [34]. Recent results suggest that the lipid composition of the target membrane is important for transduction activity. We have directly observed TAT peptide entry into giant unilamellar vesicles without any endocytotic machinery, provided that the target membranes have negative intrinsic curvature lipids (such as those with PE head groups) at concentrations above a minimum 'threshold' concentration [57]. This has also been observed with Penetratin, which traverses lipid bilayers containing PE lipids [58].

A number of studies have investigated the question of how cell-penetrating peptides dissociate from the plasma membrane once they are internalized. For example, it has been shown that the arginine-rich peptide,  $\text{R}_8\text{W}$ , can strongly interact with the anionic cellular cytoskeleton components actin and tubulin, leading to aggregation [59]. The TAT peptide also binds to and condenses DNA. Moreover, the binding constant for the TAT peptide with DNA is 1–2 orders of magnitude higher than for heparin sulfate, leading to the hypothesis that high DNA-binding affinity could facilitate the release of cargo after cellular uptake [60], by promoting competition for the membrane association of polycationic peptides.

The debate on the mechanism of cell-penetrating peptides, mostly notably TAT, has often been characterized by a type of ‘either/or’ discourse, such as the whether direct translocation or some specific form of endocytosis is most relevant. Since the cellular uptake of arginine-rich peptides is dependent on a variety of factors, including temperature, incubation time, cell type, cargo type and size, linkage type and size [4,61], comparison between different experiments have been difficult, and has compounded the controversy surrounding the uptake mechanism. It has no doubt been recognized that more than one mechanism may be involved in TAT translocation activity. This recognition accommodates the broad range of proposed hypotheses, and can potentially conclude the discourse with an artificial consensus. However, an acknowledgement of multiple mechanisms of entry does not explain the central phenomenon: How does a relatively simple molecule like TAT facilitate mechanisms as different as direct entry and the multiple endocytotic mechanisms? Rather than debate the differences between the distinct observed mechanisms, we focus on what these different mechanisms have in common, and relate these common features to what the physical chemistry of cell-penetrating peptides allows them to do.

## 4. Peptide–membrane interactions

### 4.1. Electrostatics

Electrostatics in aqueous environments is counterintuitive. At physiological conditions, electric fields from cell-penetrating peptides are strongly reduced by the large dielectric constant of water, and by screening from ions from dissociated salts. That is not to say that electrostatic interactions are weak. The entropy of ions can result in strong interactions between charged objects in water despite short screening lengths, via coupling between osmotic and electrostatic interactions. For example, the attraction between a cationic cell-penetrating peptide and an anionic membrane is driven by the entropy gain from release of condensed counterions. A flat, charged membrane is covered on both sides by a layer of condensed counterions (the ‘Gouy–Chapman’ layer), consistent with the non-linear Poisson–Boltzmann equation [62]. A similar analysis reveals that charged polymers are also coated by a layer of condensed counterions (the ‘Manning layer’) [63]. In the present case of cell-penetrating peptide adsorption onto an oppositely charged membrane, the complementary charge distributions can electrostatically compensate one another. This means that condensed counterions are no longer needed by the membrane and the polymer at the regions of contact, and thus can be released for a large entropic gain. The sequence of events outlined above leads to a strong electrostatic attraction. This type of electrostatic interaction has been observed for self-assembled complexes between membranes and a variety of anionic polymers, including DNA [64–66], F-actin [67], microtubules [68], and filamentous phages [69].

### 4.2. Induced membrane deformation

In general, the trade-off between electrostatic interactions and membrane deformations control the interactions between charged polymers and membrane systems [64,65,67,68]. Since counterion release is maximized when the cationic lipids are closely associated with the anionic charges on a polymer or an oligomer, electrostatic binding favors a state where the membrane wraps around a charged oligomer, which leads to maximal contact and maximal entropy gain from counterion release. However, the wrapped configuration is resisted by the elastic cost of deforming the membrane. The energetic penalty from membrane curvature

distortions is usually described using the treatment introduced by Helfrich [70].

The curvature elastic energy per unit area of bending a membrane is given by:

$$f = 2\kappa(H - c_0)^2 + \kappa_G K$$

$$\text{where } H = \frac{1}{2}c_1 + c_2, \quad K = c_1c_2$$

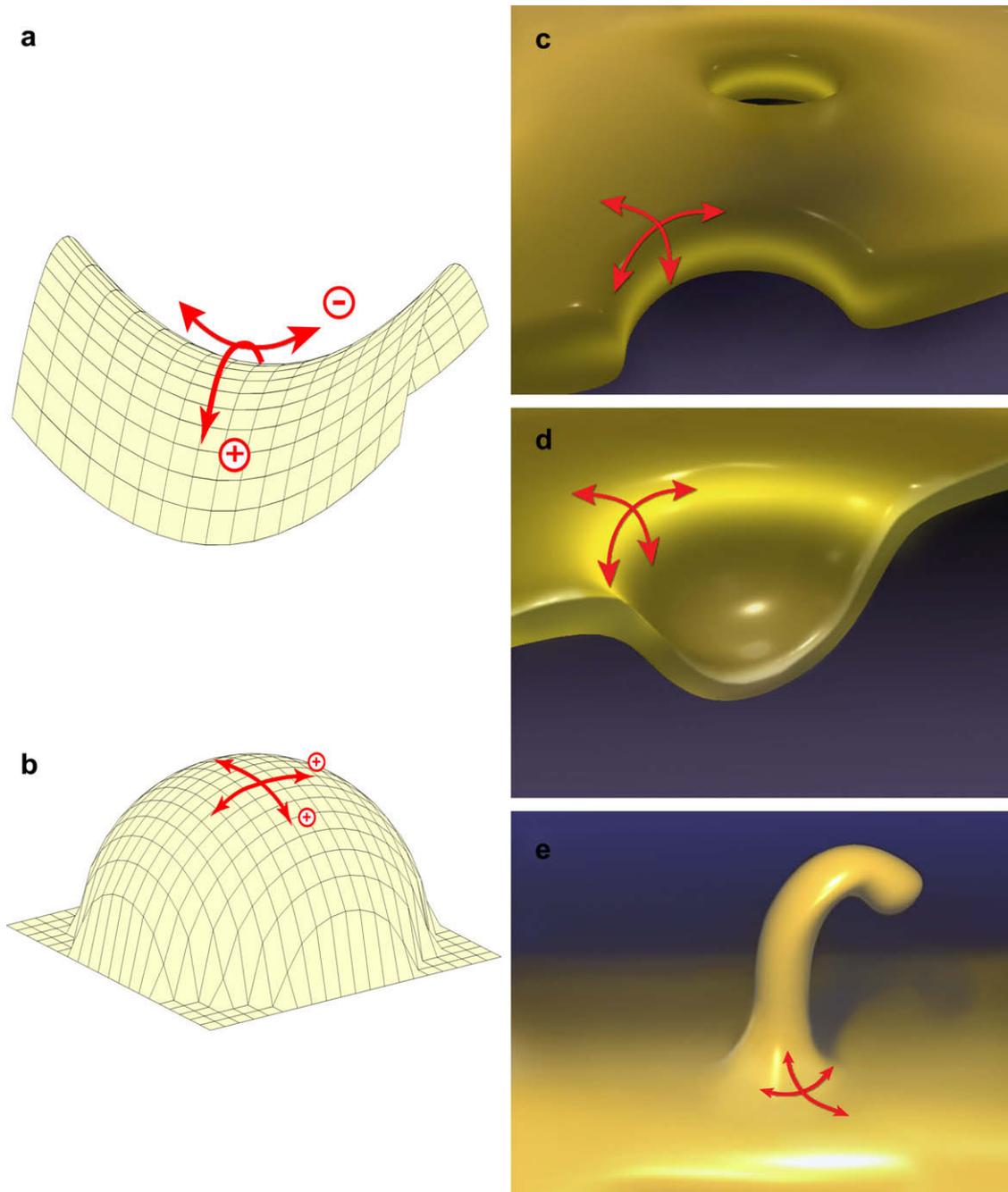
Here  $c_1$  and  $c_2$  are the principal curvatures at a point on the surface. Geometrically, at any point on the surface, we can define  $c_1 = 1/R_1$ , and  $c_2 = 1/R_2$  where  $R_1$  and  $R_2$  are the principle radii of curvature; the radii of circles constructed which best fit the minimum and maximum curvatures at the point on the surface. For smooth surfaces  $c_1$ , and  $c_2$  are in perpendicular directions. The mean curvature is defined by  $H$ , and is a measure of the degree of membrane bending. (For example, since  $c_1 = 1/R_1$ : when the curvature  $c_1$  is large,  $R_1$  is small, and implies a tight bend.) The Gaussian curvature,  $K$ , is defined by the product of  $c_1$  and  $c_2$ . This type of curvature is related to the topological changes of the membrane, such as pore formation. (More on this later.) The mean curvature that minimizes the free energy,  $c_0$ , is called the ‘intrinsic curvature’ of the membrane. The bending modulus,  $\kappa$ , is the energy cost of deviating from the spontaneous curvature and the Gaussian curvature modulus,  $\kappa_G$ , measures the energy cost of topological changes [71,72]. This last quantity is an important parameter that governs a membrane’s tendency for pore formation, for example.

In accord with intuition, the intrinsic curvature  $c_0$  of a membrane depends on the shape of the lipid molecule. For  $c_0 < 0$ , the lipids are shaped like traffic cones, with small head groups and bulky tails, and tend to bend toward the hydrophilic side. For  $c_0 > 0$ , the lipids are shaped like ice-cream cones, with bulky head groups and small tails, and tend to bend toward the hydrophobic side. Lipids such as zwitterionic dioleoylphosphatidylcholine (DOPC), cationic dioleoyltrimethylammonium-propane (DOTAP), and anionic dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylserine (DOPS), have head group areas approximately the same as their tails and therefore have zero intrinsic curvature. They assemble to form flat membranes. Conversely, dioleoylphosphatidylethanolamine (DOPE) lipids have a head group smaller than their tail region, giving them negative intrinsic curvature [73].

## 5. Significance of Gaussian membrane curvature for pore formation and other cellular processes

It can be seen that formation of a pore in a membrane requires the generation of negative Gaussian curvature ( $K < 0$ ). Illustration of this point can be seen by recalling that  $K$  at a point on the membrane is the product of the two principle axes of curvature at that point. Therefore,  $K < 0$  implies the principle axes of curvature at a given point must curve in opposite directions, so that the membrane is locally shaped like a saddle (Fig. 1a). This is the type of curvature seen objects with holes, for example a torus: the ‘hole’ of the torus is composed of regions with saddle-shaped curvature. In contrast, objects such as spheres have no holes, and have  $K > 0$  everywhere on their surface (Fig. 1b).

In a more general compass, it can be seen that negative Gaussian curvature is broadly enabling. While negative Gaussian curvature is topologically necessary for pore formation (Fig. 1c) it can be seen in other processes. From Fig. 1, it can be seen that generation of negative Gaussian membrane curvature is a necessary condition for the dimples for caveoli-based endocytosis, for the cytoskeleton-driven protrusions in macropinocytosis, as well as for pore formation. The Gauss–Bonnet theorem shows that if pores do not form, the net change in the global Gaussian curvature of the membrane is zero, i.e.  $\Delta \int K \cdot dA = 0$ : if one region of the membrane develops



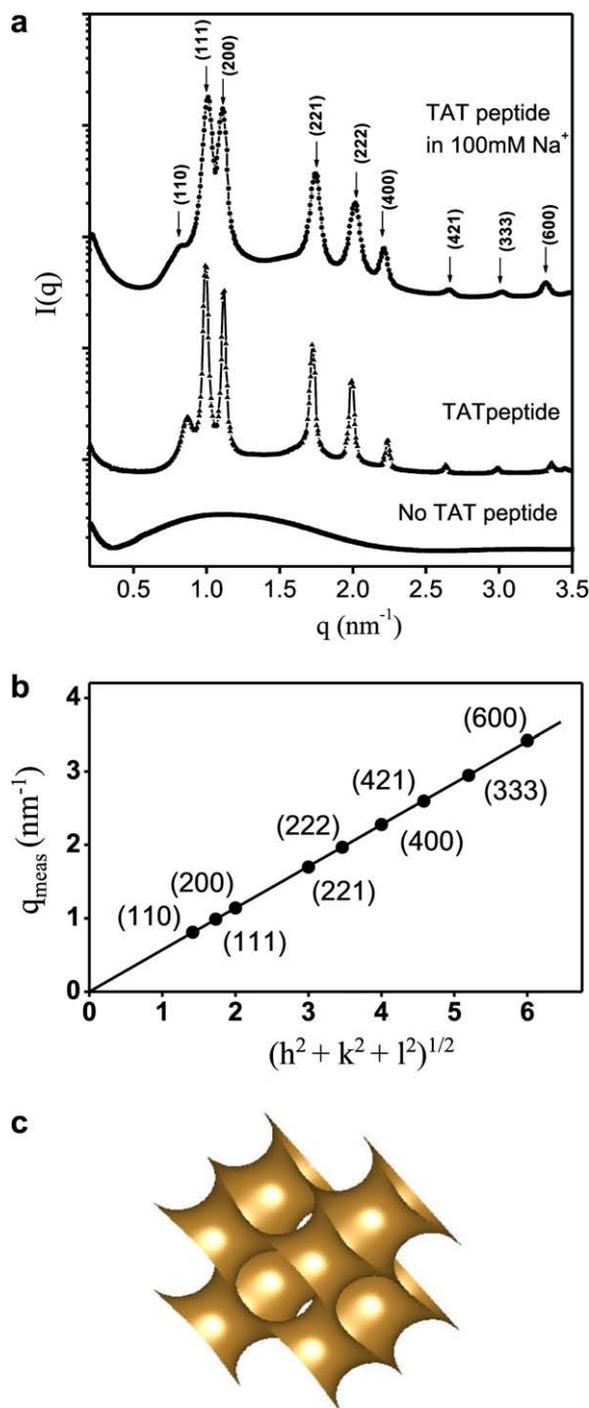
**Fig. 1.** Examples of (a) negative Gaussian curvature and (b) positive Gaussian curvature. The former is saddle shaped and found in objects with holes such as a donut. The latter is found on objects like spheres or ellipsoids, and do not have holes. Negative Gaussian curvature is topologically necessary for the formation of (c) membrane pores, (d) membrane dimples or invaginations (such as those in endocytosis), and (e) membrane protrusions (such as those in macropinocytosis).

positive Gaussian curvature then some other region will develop negative Gaussian curvature to exactly compensate for the distortion. Examples of this balance of curvatures constrained by topology can be seen in proposed types of TAT entry mechanisms. The invaginations in a cell's plasma membrane surface during endocytosis display negative Gaussian curvature along the rim of the enclosure (Fig. 1d) while the 'pocket' is sphere-shaped with positive Gaussian curvature. For cellular uptake processes such as macropinocytosis, the positive Gaussian curvature at the tip of protrusions is countered by the negative Gaussian curvature found at the base of the extension (Fig. 1e). Now that we see how these negative Gaussian membrane distortions are implicated in direct pore formation, invaginations from endocytosis, protrusions from macr-

opinocytosis, the next question is, what is the relation between this type of curvature and cell-penetrating peptides.

## 6. The arginines in the TAT peptide generate negative Gaussian curvature

In our recent work, we have shown that the HIV TAT cell-penetrating peptide generates negative Gaussian membrane curvature, the type of membrane curvature found in pores, protrusions from macropinocytosis, invaginations from endocytosis [57]. Using synchrotron small angle X-ray scattering (SAXS), we demonstrated that the TAT peptide (amino acid sequence: YGRKKRRQRRR)



**Fig. 2.** Synchrotron X-ray diffraction studies show that the HIV TAT cell-penetrating peptide induces negative Gaussian curvature on membranes. This structural tendency can lower the free energy barrier for a broad range of different entry mechanisms.

generates a cubic Pn3m phase in membranes enriched with negative intrinsic curvature lipids. This structural tendency to form a cubic phase can in fact be observed in other agents in different biological contexts. For example, under specific lipid compositions and solution conditions, pore forming antimicrobial peptides alamethicin [74], gramicidin S [75,76], lactoferricin (LF11) derived peptides, VS1–13 and VS1–24 [77], as well as protegrin-1 and pepitidyl-glycylleucine-carboxyamide [78] will also induce cubic phases. It is interesting to note that the negative Gaussian curva-

ture necessary to form these observed cubic phases is also the structural ingredient topologically required for pore formation.

Before addition of TAT peptide a broad diffraction feature is seen (Fig. 2a, bottom). This is consistent with the form factor of a lipid bilayer indicating the presence small unilamellar vesicles. After addition of TAT peptide, the spectra display correlation peaks (Fig. 2a, middle) which show good agreement with those expected for a cubic Pn3m 'double-diamond' lattice (Fig. 2b), which is rich in negative Gaussian curvature. The cubic Pn3m is a bicontinuous phase characterized by two non-intersecting tetrahedral networks of water channels separated by a lipid bilayer [79]. The bilayer surface between membrane leaflets has zero mean curvature but negative Gaussian curvature (Fig. 2c). By fitting the slope of the measured peak positions we calculate a lattice spacing of  $a = 10.97$  nm, for TAT peptide. Similar features are seen in the presence of 100 mM NaCl indicating that TAT peptide can generate these dramatic changes in membrane topology at physiological salt conditions (Fig. 2a, top).

If the generation of negative Gaussian curvature is correlated with cell-penetrating peptide permeation ability, then peptides that are unable to penetrate cells should not induce phases with negative Gaussian curvature in membranes. Experiments with polylysine, K<sub>8</sub>, on membranes of identical composition to those used for TAT peptide, show interactions between the lipid head groups and polylysine induce wrapping of the membrane monolayers [57]. The result is an inverted hexagonal phase where the much smaller columnar channels are filled with peptide and solution. This phase has negative mean curvature but zero Gaussian curvature. The absence of negative Gaussian curvature may explain the poor transduction ability of polylysine.

Comparison of arginine and lysine side chains reveals the molecular origins responsible for differences in peptide interaction with lipid membranes. Arginine is the most basic of all amino acids, because its side chain ends with a guanidinium group. Structurally guanidinium is characterized by a planar Y-shape which acts to delocalize its cationic charge. The result is a moiety with six potential hydrogen bonding sites. The multiple hydrogen bonding abilities as well as its unique shape allow a guanidinium group to direct both electrostatic and hydrogen bonding with anionic and polar molecules [80]. When arginine interacts with phospholipids this takes the form of bi- or multi-dentate hydrogen bonding from simultaneous association with the phosphates of more than one lipid head group. However, lysine has an amino group which only forms monodentate hydrogen bonds and therefore interacts with the phosphate on a single lipid head group. In other words, guanidinium is more efficient at interacting with bulky lipid head groups. It is well known that this can lead to buckling of the peptide and generate positive curvature along its length. Since the effect of a cationic peptide on an anionic membrane is create a tendency for the membrane to wrap around the peptide, both polyarginine and polylysine can generate negative mean curvature perpendicular to their length. However, since only arginine can form bi-dentate hydrogen bonds, an arginine-rich cell-penetrating peptide can bond with more zwitterionic and anionic lipids and therefore generate positive curvature along its contour length, thus resulting in negative Gaussian curvature [81], which is manifested in cell-penetrating peptide generation of the Pn3m cubic phase. In addition to HIV TAT peptide, experiments with other arginine-rich cell-penetrating peptides ANTP Penetratin and polyarginine also show similar behavior [82].

The structural tendency of a membrane to form negative Gaussian curvature under the influence of TAT cell-penetrating peptide has a significant dependence on membrane curvature. This may explain the range of observed results ranging from strong translocation activity to no translocation activity in biophysical experiments. For example, X-ray diffraction data show a strong

dependence on membrane lipid composition and phase behavior. The Pn3m cubic phase is generated by TAT peptide in membranes enriched with negative intrinsic curvature lipids. Substitution of DOPE ( $c_0 < 0$ ) with DOPC ( $c_0 = 0$ ) makes the intrinsic curvature of the membrane monolayers less negative which results in the loss of the negative Gaussian curvature-rich Pn3m phase. Moreover, direct translocation of TAT peptide into the interior of giant unilamellar vesicles (GUVs) was likewise shown to depend on a minimum threshold amount of membrane PE content [57]. As another example, high cholesterol membrane content has been shown to accompany each type of receptor-independent endocytotic pathway implicated in cell-penetrating peptide uptake, including lipid raft-mediated caveolae and macropinocytosis [32,83] as well as clathrin coated pit endocytosis [84]. We find that the presence of cholesterol at typical eukaryotic values will drastically enhance the ability of a membrane to form negative Gaussian curvature necessary for these mechanisms [82].

## 7. Outlook

In this brief synopsis, we have reviewed some of the representative work on the mechanism of cell-penetrating peptides. Rather than focusing on differences between the distinct observed mechanisms of entry (such as direct translocation, and various endocytotic mechanisms) we have instead concentrated on what these different mechanisms have in common in terms of membrane topology, and related these common features to what the physical chemistry of cell-penetrating peptides allows them to do. We find that negative Gaussian membrane curvature is broadly enabling. The induction of such curvature can lower the free energy barriers for a range of different entry mechanisms, such as direct translocation as well as endocytotic pathways. Indeed, it is possible that cell-penetrating peptides interact with cells in other important ways besides induction of membrane curvature. For example, one of the neglected biophysical aspects of TAT is its high positive charge density. TAT can have strong electrostatic interactions with different components of the cell besides the cell membrane, in ways that contributes to its activity. Useful reviews of electrostatic interactions in biological physics include [85–90].

## Acknowledgements

We thank the NSF (DMR-0804363, CBET-08429293, RPI-UIUC NSEC) for support.

## References

- Anderson, W.F. (1998) Human gene therapy. *Nature* 392, 25–30.
- Luo, D. and Saltzman, W. (2000) Synthetic DNA delivery systems. *Nat. Biotech.* 18, 33–37.
- El-Sayed, A., Futaki, S. and Harashima, H. (2009) Delivery of macromolecules using arginine-rich cell-penetrating peptides: ways to overcome endosomal entrapment. *AAPS J.* 11, 13–22.
- Wender, P.A., Galliher, W.C., Goun, E.L., Jones, L.R. and Pillow, T.H. (2008) The design of guanidinium-rich transporters and their internalization mechanisms. *Adv. Drug Deliv. Rev.* 60, 452–472.
- Wadia, J. and Dowdy, S. (2005) Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. *Adv. Drug Deliv. Rev.* 57, 579–596.
- Green, M. and Loewenstein, P.M. (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus TAT trans-activator protein. *Cell* 55, 1179–1188.
- Frankel, A.D. and Pabo, C.O. (1988) Cellular uptake of the TAT protein from human immunodeficiency virus. *Cell* 55, 1189–1193.
- Jeang, K., Xiao, H. and Rich, E. (1999) Multifaceted activities of the HIV-1 transactivator of transcription, TAT. *J. Biol. Chem.* 274, 28837–28840.
- Vives, E., Brodin, P. and Lebleu, B. (1997) A truncated HIV-1 TAT protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 272, 16010–16017.
- Torchilin, V., Rammohan, R., Weissig, V. and Levchenko, T. (2001) TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci.* 98, 8786–8791.
- Ziegler, A., Blatter, X., Seelig, A. and Seelig, J. (2003) Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis. *Biochemistry* 42, 9185–9194.
- Goncalves, E., Kitas, E. and Seelig, J. (2005) Binding of oligoarginine to membrane lipids and heparan sulfate: structural and thermodynamic characterization of a cell-penetrating peptide. *Biochemistry* 44, 2692–2702.
- Ziegler, A. (2008) Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans. *Adv. Drug Deliv. Rev.* 60, 580–597.
- Caesar, C., Esbjorn, E., Lincoln, P. and Norden, B. (2006) Membrane interactions of cell-penetrating peptides probed by tryptophan fluorescence and dichroism techniques: correlations of structure to cellular uptake. *Biochemistry* 45, 7682–7692.
- Magzoub, M., Eriksson, L. and Graslund, A. (2002) Conformational states of the cell-penetrating peptide penetratin when interacting with phospholipid vesicles: effects of surface charge and peptide concentration. *Biochim. Biophys. Acta* 1563, 53–63.
- Christiaens, B., Symoens, S., Vanderheyden, S., Engelborghs, Y., Joliet, A., Prochiantz, A., Vandekerckhove, J., Rosseneu, M. and Vanloo, B. (2002) Tryptophan fluorescence study of the interaction of penetratin peptides with model membranes. *Eur. J. Biochem.* 269, 2918–2926.
- Dom, G., Shaw-Jackson, C., Matis, C., Bouffieux, O., Picard, J., Prochiantz, A., Mingeot-Leclercq, M., Brasseur, R. and Rezsosahy, R. (2003) Cellular uptake of Antennapedia Penetratin peptides is a two-step process in which phase transfer precedes a tryptophan-dependent translocation. *Nucl. Acid Res.* 31, 556–561.
- Binder, H. and Lindblom, G. (2003) Charge-dependent translocation of the Trojan peptide penetratin across lipid membranes. *Biophys. J.* 85, 982–995.
- Schwarze, S.R., Hruska, K.A. and Dowdy, S.F. (2000) Protein transduction: unrestricted delivery into all cells? *Trends Cell Biol.* 10, 290–295.
- Mitchell, D.J., Kim, D.T., Steinman, L., Fathman, C.G. and Rothbard, J.B. (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. *J. Pept. Res.* 56, 318–325.
- Wender, P.A., Mitchell, D.J., Pattabiraman, K., Pelkey, E.T., Steinman, L. and Rothbard, J.B. (2000) The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptidic molecular transporters. *Proc. Natl. Acad. Sci.* 97, 13003–13008.
- Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K. and Sugiura, Y. (2001) Arginine-rich peptides: an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* 276, 5836–5840.
- Wender, P., Rothbard, J., Jessop, T., Kreider, E. and Wylie, B. (2002) Oligocarbamate molecular transporters: design, synthesis, and biological evaluation of a new class of transporters for drug delivery. *J. Am. Chem. Soc.* 124, 13382–13383.
- Wender, P., Kreider, E., Pelkey, E., Rothbard, J. and VanDeusen, C. (2005) Dendritic molecular transporters: synthesis and evaluation of tunable polyguanidino dendrimers that facilitate cellular uptake. *Org. Lett.* 7, 4815–4818.
- Elson-Schwab, L., Garner, O., Schuksz, M., Crawford, B., Esko, J. and Tor, Y. (2007) Guanidinylated neomycin delivers large, bioactive cargo into cells through a heparan sulfate-dependent pathway. *J. Biol. Chem.* 282, 13585–13591.
- Holowka, E.P., Sun, V.Z., Kamei, D.T. and Deming, T.J. (2007) Polyarginine segments in block copolypeptides drive both vesicular assembly and intracellular delivery. *Nat. Mater.* 6, 52–57.
- Richard, J.P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M.J., Chernomordik, L.V. and Lebleu, B. (2003) Cell-penetrating peptides: a reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* 278, 585–590.
- Fischer, R., Fotin-Mleczek, M., Hufnagel, H. and Brock, R. (2005) Break on through to the other side-biophysics and cell biology shed light on cell-penetrating peptides. *Chem. Biol. Chem.* 6, 2126–2142.
- Drin, G., Cottin, S., Blanc, E., Rees, A. and Tamsamani, J. (2003) Studies on the internalization mechanism of cationic cell-penetrating peptides. *J. Biol. Chem.* 278, 31192–31201.
- Nakase, I., Niwa, M., Takeuchi, T., Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S., Ueda, K., Simpson, J.C., Jones, A.T., Sugiura, Y. and Futaki, S. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol. Ther.* 10, 1011–1022.
- Fretz, M., Koning, G., Mastrobattista, E., Jiskoot, W. and Storm, G. (2004) OVCAR-3 cells internalize TAT-peptide modified liposomes by endocytosis. *Biochim. Biophys. Acta* 1665, 48–56.
- Wadia, J., Stan, R. and Dowdy, S.F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* 10, 310–315.
- Richard, J.P., Melikov, K., Brook, H., Prevot, P., Lebleu, B. and Chernomordik, L.V. (2005) Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J. Biol. Chem.* 280, 15300–15306.
- Tiriveedhi, V. and Butko, P. (2007) A fluorescence spectroscopy study on the interactions of the TAT-PTD peptide with model lipid membranes. *Biochemistry* 46, 3888–3895.
- Ziegler, A. and Seelig, J. (2008) Binding and clustering of glycosaminoglycans: a common property of mono- and multivalent cell-penetrating compounds. *Biophys. J.* 94, 2142–2149.

- [36] Rusnati, M., Coltrini, D., Oreste, P., Zoppetti, G., Albini, A., Noonan, D., Fagagna, F., Giacca, M. and Prestai, M. (1997) Interaction of HIV-1 TAT protein with heparin. *J. Biol. Chem.* 10, 11313–11320.
- [37] Hakansson, S., Jacobs, A. and Caffrey, M. (2001) Heparin binding by the HIV-1 TAT protein transduction domain. *Protein Sci.* 10, 2138–2139.
- [38] Ziegler, A., Nervi, P., Durrenberger, M. and Seelig, J. (2005) The cationic cell-penetrating peptide CPP(TAT) derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical and metabolic evidence. *Biochemistry* 44, 138–148.
- [39] Console, S., Marty, C., Garcia-Echeverria, C., Schwendener, R. and Ballmer-Hofer, K. (2003) Antennapedia and HIV transactivator of transcription (TAT) "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J. Biol. Chem.* 278, 35109–35114.
- [40] Sandgren, S., Cheng, F. and Belting, M. (2002) Nuclear targeting of macromolecular polyanions by an HIV-TAT derived peptide. Role for cell-surface proteoglycans. *J. Biol. Chem.* 277, 38877–38883.
- [41] Ferrari, A., Pellegrini, V., Arcangeli, C., Fittipaldi, A., Giacca, M. and Beltram, F. (2003) Caveolae-mediated internalization of extracellular HIV-1 TAT fusion proteins visualized in real time. *Mol. Ther.* 8, 284–294.
- [42] Tyagi, M., Rusnati, M., Presta, M. and Giacca, M. (2001) Internalization of HIV-1 TAT requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 276, 3254–3261.
- [43] Khalil, I., Kogure, K., Akita, H. and Harashima, H. (2006) Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol. Rev.* 58, 32–45.
- [44] Potocky, T., Menon, A. and Gellman, S. (2003) Cytoplasmic and nuclear delivery of a TAT-derived peptide and a B-peptide after endocytic uptake into HeLa cells. *J. Biol. Chem.* 278, 50188–50194.
- [45] Jones, S., Christison, R., Bundell, K., Joyce, C., Brockbank, S., Newham, P. and Lindsay, M. (2005) Characterisation of cell-penetrating peptide-mediated peptide delivery. *Brit. J. Pharmacol.* 145, 1093–1102.
- [46] Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F. and Giacca, M. (2003) Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 TAT fusion proteins. *J. Biol. Chem.* 278, 34141–34149.
- [47] Säälk, P., Elmquist, A., Hansen, M., Padari, K., Saar, K., Viht, K., Langel, U. and Pooga, M. (2004) Protein cargo delivery properties of cell-penetrating peptides. A comparative study. *Bioconjugate Chem.* 15, 1246–1253.
- [48] Kaplan, I.M., Wadia, J.S. and Dowdy, S.F. (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J. Control. Release* 102, 247–253.
- [49] Zaro, J., Rajapaksa, T., Okamoto, C. and Shen, W. (2006) Membrane transduction of oligoarginine in HeLa cells is not mediated by macropinocytosis. *Mol. Pharmacol.* 3, 181–186.
- [50] Maiolo, J., Ferrer, M. and Ottinger, E. (2005) Effects of cargo molecules on the cellular uptake of arginine-rich cell-penetrating peptides. *Biochim. Biophys. Acta* 1712, 161–172.
- [51] Ter-Avetisyan, G., Tunnemann, G., Nowak, D., Nitschke, M., Herrmann, A., Drab, M. and Cardoso, M.C. (2009) Cell entry of arginine-rich peptides is independent of endocytosis. *J. Biol. Chem.* 284, 3370–3378.
- [52] Iwasa, A., Akita, H., Khalil, I., Kogure, K., Futaki, S. and Harashima, H. (2006) Cellular uptake and subsequent intracellular trafficking of R8-liposomes introduced at low temperature. *Biochim. Biophys. Acta* 1758, 713–720.
- [53] Fretz, M.M., Penning, N., Al-Taei, S., Futaki, S., Takeuchi, T., Nakase, I., Storm, G. and Jones, A.T. (2007) Temperature-, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells. *Biochem. J.* 403, 335–342.
- [54] Thoren, P., Persson, D., Lincoln, P. and Norden, B. (2005) Membrane destabilizing properties of cell-penetrating peptides. *Biophys. Chem.* 114, 169–179.
- [55] Drin, G., Demene, H., Tamsamani, J. and Brasseur, R. (2001) Translocation of the pAntp peptide and its amphipathic analogue AP-2AL. *Biochemistry* 40, 1824–1834.
- [56] Persson, D., Thoren, P., Herner, M., Lincoln, P. and Norden, B. (2003) Application of a novel analysis to measure the binding of the membrane-translocating peptide penetratin to negatively charged liposomes. *Biochemistry* 42, 421–429.
- [57] Mishra, A., Gordon, V., Yang, L., Coridan, R. and Wong, G. (2008) HIV TAT forms pores in membranes by inducing saddle-splay curvature: potential role of bidentate hydrogen bonding. *Angew. Chem., Int. Ed.* 47, 2986–2989.
- [58] Thoren, P.E.G., Persson, D., Karlsson, M. and Norden, B. (2000) The Antennapedia peptide penetratin translocates across lipid bilayers – the first direct observation. *FEBS Lett.* 482, 265–268.
- [59] Jones, L., Yazzie, B. and Middaugh, C. (2004) Polyanions and the proteome. *Mol. Cell Proteom.* 3, 746–769.
- [60] Ziegler, A. and Seelig, J. (2007) High affinity of the cell-penetrating peptide HIV-1 TAT-PTD for DNA. *Biochemistry* 46, 8138–8145.
- [61] Brooks, H., Lebleu, B. and Vives, E. (2005) TAT peptide-mediated cellular delivery: back to basics. *Adv. Drug Deliv. Rev.* 57, 559.
- [62] Zimm, B. and Bret, M. (1983) Counter-ion condensation and system dimensionality. *J. Biomol. Struct. Dynam.* 1, 461–471.
- [63] Manning, G.S. (1969) Limiting laws and counterion condensation in polyelectrolyte solutions. I. Colligative properties. *J. Chem. Phys.* 51, 924–933.
- [64] Raedler, J.O., Koltover, I., Salditt, T. and Safinya, C.R. (1997) Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science* 275, 810–814.
- [65] Koltover, I., Salditt, T., Raedler, J.O. and Safinya, C.R. (1998) An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science* 281, 78–81.
- [66] Liang, H., Angelini, T., Ho, J., Braun, P. and Wong, G. (2003) Molecular imprinting of biomineralized CdS nanostructures: crystallographic control using self-assembled DNA-membrane templates. *J. Am. Chem. Soc.* 125, 11786–11787.
- [67] Wong, G., Tang, J., Lin, A., Li, Y., Janmey, P. and Safinya, C. (2000) Hierarchical self-assembly of F-actin and cationic lipid complexes: stacked three-layer tubule networks. *Science* 288, 2035–2039.
- [68] Raviv, U., Needleman, D.J., Li, Y., Miller, H.P., Wilson, L. and Safinya, C. (2005) Cationic liposome-microtubule complexes: pathways to the formation of two-state lipid-protein nanotubes with open or closed ends. *Proc. Natl. Acad. Sci.* 102, 11167–11172.
- [69] Yang, L., Liang, H., Angelini, T., Butler, J., Coridan, R., Tang, J. and Wong, G. (2004) Self-assembled virus-membrane complexes. *Nat. Mater.* 3, 615–619.
- [70] Helfrich, W. (1973) Elastic properties of lipid bilayers: theory and possible experiments. *Naturforsch* 28C, 693–703.
- [71] Safran, S.A. (1999) Curvature elasticity of thin films. *Adv. Phys.* 48, 395–448.
- [72] Parthasarathy, R. and Groves, J. (2007) Curvature and spatial organization in biological membranes. *Soft Matter* 3, 24–33.
- [73] Zimmerberg, J. and Kozlov, M. (2006) How proteins produce cellular membrane curvature. *Nat. Rev. Mol. Cell. Biol.* 7, 9–19.
- [74] Keller, S., Gruner, S. and Gawrisch, K. (1996) Small concentrations of alamethicin induce a cubic phase in bulk phosphatidylethanolamine mixtures. *Biochim. Biophys. Acta* 1278, 241–246.
- [75] Prenner, E.J., Lewis, R.N.A.H., Neuman, K.C., Gruner, S.M., Kondejewski, L.H., Hodges, R.S. and McElhaney, R.N. (1997) Nonlamellar phases induced by the interaction of gramicidin S with lipid bilayers. A possible relationship to membrane-disrupting activity. *Biochemistry* 36, 7906–7916.
- [76] Staudegger, E., Prenner, E.J., Kriechbaum, M., Degovics, G., Lewis, R.N.A.H., McElhaney, R.N. and Lohner, K. (2000) X-ray studies on the interaction of the antimicrobial peptide gramicidin S with microbial lipid extracts: evidence for cubic phase formation. *Biochim. Biophys. Acta* 1468, 213–230.
- [77] Zweytick, D., Tumer, S., Blondelle, S.E. and Lohner, K. (2008) Membrane curvature stress and antibacterial activity of lactoferricin derivatives. *Biochem. Biophys. Res. Commun.* 369, 395–400.
- [78] Hickel, A., Danner-Pongratz, S., Amenitsch, H., Degovics, G., Rappolt, M., Lohner, K. and Pabst, G. (2008) Influence of antimicrobial peptides on the formation of nonlamellar lipid mesophases. *Biochim. Biophys. Acta* 1778, 2325–2333.
- [79] So, P., Gruner, S. and Erramilli, S. (1993) Pressure-induced topological phase transitions in membranes. *Phys. Rev. Lett.* 70, 3455–3458.
- [80] Schug, K.A. and Linder, W. (2005) Noncovalent binding between guanidinium and anionic groups: focus on biological- and synthetic-based arginine/guanidinium interactions with phosph[on]ate and sulf[on]ate residues. *Chem. Rev.* 105, 67–113.
- [81] Rothbard, J.B., Jessop, T.C. and Wender, P.A. (2005) Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells. *Adv. Drug Deliv. Rev.* 57, 495–504.
- [82] Mishra, A., Lai, G., Schmidt, N.W. and Wong, G.C.L. To be published.
- [83] Conner, S.D. and Schmid, S.L. (2003) Regulated portals of entry into the cell. *Nature* 422, 37–44.
- [84] Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M., Keen, J. and McGraw, T. (1999) Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci.* 96, 6775–6780.
- [85] Wong, G. (2006) Electrostatics of rigid polyelectrolytes. *Curr. Opin. Colloid Interf. Sci.* 11, 310–315.
- [86] Grosberg, A.Y., Nguyen, T.T. and Shklovskii, B.I. (2002) Colloquium: the physics of charge inversion in chemical and biological systems. *Rev. Mod. Phys.* 74, 329.
- [87] Levin, Y. (2002) Electrostatic correlations: from plasma to biology. *Rep. Prog. Phys.* 1577.
- [88] Gelbart, W.M., Bruinsma, R.F., Pincus, P.A. and Parsegian, V.A. (2000) DNA-inspired electrostatics. *Phys. Today* 53, 38–44.
- [89] Holm, C. and Podgornik, R. (2001) Electrostatic Effects in Soft Matter and Biophysics, Kluwer, Dordrecht.
- [90] Wong, G.C.L. and Pollack, L. (2009) Electrostatics of strongly charged biological polymers. *Ann. Rev. Phys. Chem.*, accepted for publication.